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Improved Medium for Detection of Citrate-Fermenting *Streptococcus lactis* subsp. *diacetylactis*†

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A modified medium which distinguished between citrate-fermenting and non-citrate-fermenting species of lactic streptococci within 48 h was developed. In addition, the occurrence of citrate-negative variants in citrate-positive populations of *Streptococcus lactis* subsp. *diacetylactis* could be detected.

The flavor and aroma of many fermented dairy products are dependent upon organisms which synthesize diacetyl. The specific requirement of citrate as a precursor in diacetyl synthesis in *Streptococcus diacetylactis* and *Leuconostoc* species has been well established (1, 4). Uptake of citrate is mediated by a citrate permease system (3, 11). Once inside the cell, citrate is broken down by the enzyme citritase to oxaloacetate and acetic acid (2). Oxaloacetate is converted to pyruvate, which, when in excess, is converted through a series of intermediate reactions to diacetyl (8, 11). The characteristic dependency of cells on a citrate permease system for eventual diacetyl synthesis has been used in several media designed to isolate aroma-producing bacteria. The need for distinguishing between citrate-fermenting and non-citrate-fermenting strains is twofold. Primarily, it is often necessary when monitoring strain balance to determine the ratio of aroma producers to non-aroma producers in a mixed starter strain. In addition, recent evidence suggests that the genes controlling citrate permease activity are associated with plasmid deoxyribonucleic acid in *S. diacetylactis* (5). Because plasmid deoxyribonucleic acid is unstable, citrate-negative variants which are unable to produce diacetyl can spontaneously occur. Thus, it would be advantageous to have a means for isolating strong citrate-fermenting organisms and to ensure that diacetyl-producing cultures are pure with respect to citrate fermentation.

To date, agar media used to distinguish citrate-fermenting from non-citrate-fermenting dairy starter organisms have contained insoluble calcium citrate, the utilization of which results in zones of clearing around colonies (9, 10). Such media have several drawbacks. They are fairly time-consuming to prepare, because agents such as carboxymethylcellulose must be added to prevent the insoluble calcium citrate from settling. In addition, colonies on the media must be well separated to assess zones of clearing, which may take 5 days or longer to develop.

In 1952, Kneteman introduced a medium which he found was successful in distinguishing citrate-fermenting from non-citrate-fermenting organisms (7). The medium involved acid whey and milk, along with solutions of potassium ferrocyanide and ferric citrate. The presence of citrate in the medium inhibited a reaction between the ferric ion and potassium ferrocyanide. Colonies able to utilize the citrate initiated a reaction between these ions resulting in formation of dark blue colonies (Prussian blue); colonies unable to utilize citrate remained white.

Undoubtedly, the complicated procedure necessary to prepare this medium limited its use. However, in our laboratory, a modification of this medium which requires little time for preparation and provides a quick and efficient means for distinguishing between citrate-fermenting and non-citrate-fermenting lactic streptococci was recently developed.

The differential medium contained 1% (wt/vol) nonfat milk (Matrix Mother Culture Medium, Galloway-West, Fond du Lac, Wis.), 0.25% milk protein-hydrolysate peptone (BBL Microbiology Systems, Cockeysville, Md.), 0.5% dextrose, and 1.5% agar. The pH was adjusted to 6.6 before sterilization. After sterilization of the medium for 12 min at 10 lb/in^2, it was tempered at 45°C. Two solutions, one containing 10% potassium ferricyanide and one containing 1 g of ferric citrate and 1 g of sodium citrate in 40 ml of water, were steamed (100°C) for 30 min. Ten milliliters of each solution was added to 1 liter of agar medium, and the agar was swirled gently and poured. Plates were dried in the dark for 24 h at 30°C.

Spread plates were made of various *Streptococcus lactis*, *Streptococcus cremoris*, and *S. diacetylactis* cultures. Plates were incubated for 48 h in a hydrogen-carbon dioxide atmosphere (GasPak System, BBL Microbiology Systems) or aerobically at 32°C. Although the various

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colony types could be distinguished on plates grown under aerobic conditions, more pronounced differentiation between citrate-fermenting and non-citrate-fermenting colonies was found when plates were grown in an anaerobic atmosphere. Colonies of *S. lactis* and *S. cremoris* were totally white in 48 h, whereas colonies of *S. diacetylactis* were Prussian blue. In addition, citrate-positive colonies of *S. diacetylactis* could be easily identified among citrate-negative variants on a 48-h spread plate (Fig. 1).

Occasional variability in the shade or amount of blue color of colonies occurred with some *S. diacetylactis* strains. This is apparently due to natural variation in the ability of different cells to transport citrate. However, all citrate-positive colonies were blue or had large blue centers. The dark blue colonies may have resulted from variants which could rapidly accumulate citrate. White colonies were confirmed to be citrate negative by King's test for diacetyl and acetoin (6).

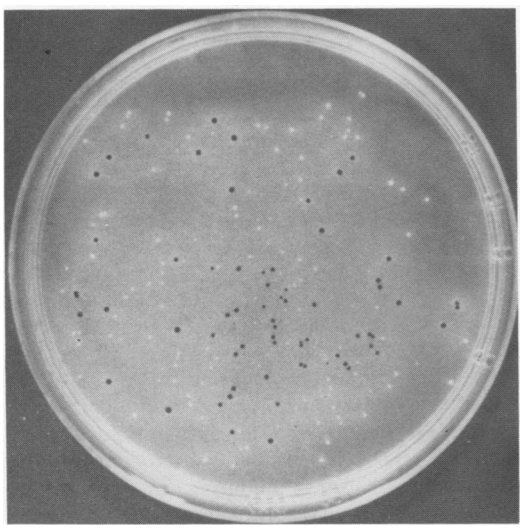

FIG. 1. *Spread plate (48 h) of a mixed culture containing S. diacetylactis 18-16 and a citrate-negative mutant, GK82 (5). Citrate-positive colonies are dark (blue on actual medium), whereas citrate-negative colonies are white.*

This medium was found exceptionally useful when attempting to isolate citrate-fermenting colonies or citrate-negative mutants. In addition, it may serve as a quick means for distinguishing *S. diacetylactis* from other lactic bacteria in mixed starter strains.

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